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Involvement of phospholipase C and intracellular calcium signaling in the gonadotropin-releasing hormone regulation of prolactin release from lactotrophs of tilapia (*Oreochromis mossambicus*)

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Abstract

Gonadotropin-releasing hormone (GnRH) is a potent stimulator of prolactin (PRL) secretion in various vertebrates including the tilapia, *Oreochromis mossambicus*. The mechanism by which GnRH regulates lactotroph cell function is poorly understood. Using the advantageous characteristics of the teleost pituitary gland from which a nearly pure population of PRL cells can be isolated, we examined whether GnRH might stimulate PRL release through an increase in phospholipase C (PLC), inositol triphosphate (IP_3), and intracellular calcium (Ca_i^{2+}) signaling. Using Ca_i^{2+} imaging and the calcium-sensitive dye fura-2, we found that chicken GnRH-II (cGnRH-II) induced a rapid dose-dependent increase in Ca_i^{2+} in dispersed tilapia lactotrophs. The Ca_i^{2+} signal was abolished by U-73122, an inhibitor of PLC-dependent phosphoinositide hydrolysis. Correspondingly, cGnRH-II-induced $tPRL_{188}$ secretion was inhibited by U-73122, suggesting that activation of PLC mediates cGnRH-II's stimulatory effect on PRL secretion. Pretreatment with 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), an inhibitor of Ca^{2+} release from intracellular stores, impeded the effect of cGnRH-II on Ca_i^{2+}. To further address the possible involvement of intracellular Ca^{2+} stores, IP_3 concentrations in the tilapia rostral pars distalis (RPD containing 95–99% PRL cells) was determined by a radioreceptor assay. We found that GnRH-II induces a rapid (<5 min) and sustained increase in IP_3 concentration in the RPD. Secretion of $tPRL_{188}$ in response to cGnRH-II was suppressed by Ca^{2+} antagonists (TMB-8 and nifedipine). These data, along with our previous findings that show PRL release increases with a rise in Ca_i^{2+}, suggest that GnRH may elicit its PRL releasing effect by increasing Ca_i^{2+}. Furthermore, the rise in Ca_i^{2+} may be derived from PLC/IP_3-induced mobilization of Ca^{2+} from intracellular stores along with influx through L-type voltage-gated Ca^{2+} channels.

Keywords: GnRH; Calcium signaling; Prolactin; Tilapia; Lactotrophs

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a potent stimulator of prolactin (PRL) secretion in various vertebrates (Blackwell et al., 1986; Robberecht et al., 1992) including the tilapia (Weber et al., 1994, 1997). In tilapia it has been shown that GnRH neurons innervate the pituitary (Yamamoto et al., 1998), and a GnRH receptor has been localized in PRL cells, which further implies the physiological importance of GnRH in the regulation of PRL release (Parhar et al., 2002). The mechanism by which GnRH regulates lactotroph cell function in tilapia and other vertebrates is, however, unknown. Activation of the transfected mammalian

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GnRH receptor has been shown to involve $G_{q/11}$ protein coupled phospholipase C (PLC) activation, increased inositol triphosphate (IP_3) production, and PRL release in GH tumor cell lines (Janovick and Conn, 1994; Ulloa-Aguirre et al., 1998). A similar induction of PLC, IP_3, and intracellular Ca^{2+} (Ca_i^{2+}) signaling occurs when two cloned native goldfish GnRH receptors are transfected in mammalian cell expression systems (Illing et al., 1999).

In mammalian gonadotrophs it has been suggested that GnRH activated IP_3-mediated Ca^{2+} release from internal stores is responsible for exocytosis (Tse et al., 1997). In these cells, parallel second messengers do not seem to contribute to GnRH-induced exocytosis, since IP_3 alone was as effective as GnRH, and because GnRH failed to trigger exocytosis when the $[Ca_i^{2+}]$ was blunted by EGTA (Tse et al., 1997).

Based on studies of GnRH-induced release of gonadotropin in mammalian pituitary cells (Hille et al., 1995; Stojilkovic and Catt, 1995), and gonadotropins and GH release in fish (Levavisivan and Yaron, 1993; Chang et al., 2000) the general mechanisms mediating GnRH actions, involve receptor activation of PLC/IP_3/diacylglycerol (DAG), Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC), mobilization of Ca^{2+} from intracellular sources, and activation of PKC. The rise in Ca^{2+}, which may be oscillatory or biphasic (gonadotropes; Johnson et al., 1999), can occur through both the influx of Ca^{2+} through VGCC and release of Ca^{2+} from IP_3-sensitive intracellular stores (gonadotropes; Mulvaney et al., 1999). Intracellular stores seem critical for the initial response in goldfish somatotropes and gonadotropes, but IP_3-sensitive pools have only been implicated for the later cell type (Johnson and Chang, 2000; Johnson et al., 2000). In tilapia PRL cells, GnRH induces a concentration-dependent increase in PRL release in accordance with a receptor-mediated effect (Weber et al., 1994, 1997) but the signaling mechanisms involved are less clear.

The objectives of the present study was to examine the putative role of Ca^{2+} signaling, PLC, IP_3, and intracellular Ca^{2+} stores in GnRH-induced stimulation of PRL release. We used the beneficial characteristics of the teleost pituitary gland from which a nearly pure population of PRL cells can be isolated from the rostral pars distalis (RPD; containing a 99% pure population of PRL cells; Nishioka et al., 1988). Using the PRL-cell model we examined: (i) the regulation of Ca_i^{2+} by cGnRH-II using image analysis of dispersed cells of the RPD and the Ca^{2+}-sensitive dye fura-2, (ii) whether PLC and intracellular Ca^{2+} pools contribute to the changes in Ca_i^{2+} elicited by cGnRH-II, (iii) whether GnRH could stimulate PLC activation and hence total IP_3 formation in tilapia RPDs, and (iv) the role of various inhibitors of PLC/Ca^{2+} signaling on GnRH-induced $tPRL_{188}$ release.

2. Materials and methods

2.1. Static incubations

Adult male tilapia (100–200 g) were maintained in freshwater at a constant photoperiod (12:12-h light–dark) for a minimum 3 weeks prior to all experiments. Fish were killed by decapitation, the RPD was dissected from the pituitary, and placed in separate wells of a Falcon 96-well plate (Becton–Dickinson, Oxnard, CA) containing 100 μl of Krebs bicarbonate–Ringers. Ringers solution contained glucose, glutamine, and Eagle's minimal essential medium (355–360 mOsm, pH 7.2; Borski et al., 1991). Tissues were incubated at 27 °C in a humidified chamber (95% O_2/5% CO_2). The chamber was continuously agitated on a gyratory platform at 60 rpm. To allow PRL release to stabilize to baseline levels (Grau et al., 1987) the tissues were pre-incubated for 2 h, after which, medium was removed and replaced with treatment medium. For the PRL release studies experiments were terminated after 30 min and 4 h, and medium and tissue were collected. Tissues were sonicated in radioimmunoassay (RIA) buffer (0.01 M sodium phosphate, 1% BSA, 0.01% NaN_3, and 0.1% Triton X-100; pH 7.3) and kept frozen at −20 °C.

2.2. PRL measurements

Release of $tPRL_{177}$ and $tPRL_{188}$ are induced at similar rates by all secretogogues examined to date, including GnRHs (Borski et al., 2001; Weber et al., 1997). Thus, in the present study it was decided to only measure the release of $tPRL_{188}$. Tilapia PRL_{188} were quantified using a homologous RIA as previously described (Ayson et al., 1993; Yada et al., 1994) and hormone release is expressed as a percentage of the total amount of hormone in the incubations (tissue + media). The measurements of $tPRL_{188}$ were conducted at the Hawaii Institute of Marine Biology (Oahu, HI).

2.3. Ca_i^{2+} measurements

PRL cells were dissociated from RPD according to previously described methods (Hyde et al., 2004). Briefly, RPD were dispersed in a Ca^{2+}-free Krebs–Ringers solution containing 0.125% (w/v) trypsin (Worthington, Lakewood, NJ) for 30 min at room temperature. Cells were centrifuged (250*g*, 5 min) and resuspended in 1 ml of Krebs–Ringers solution containing 0.125% (w/v) trypsin inhibitor (Worthington) for 10 min. Cells were washed again and re-suspended in Ca^{2+} free Krebs–Ringers solution. After an additional rinse, cells were centrifuged and re-suspended in hyperosmotic Krebs–Ringers solution (355–360 mOsm). Prolactin cells were plated onto poly-L-lysine (0.1 mg/ml)-coated glass coverslips and incubated overnight before determination of Ca_i^{2+}.

Prolactin cells were loaded with 5 μM fura-2-AM, the membrane-permeable acetoxymethyl ester derivative of fura-2 (Molecular Probes, Eugene, OR), for 90 min at 27 °C. The fura-2-AM was solubilized in anhydrous dimethyl sulfoxide (Aldrich Chemical, Milwaukee, WI) to a concentration of 5 mM before its final dilution to 5 μM. The cover slips were placed into a perifusion chamber mounted on a Zeiss Axiovert inverted microscope equipped for fluorescence. All experiments were performed using a 40×-1.3 n.a. oil objective (Zeiss). The perfusion chamber was placed on a microscope stage, and cells were continuously exposed to control medium before the start of the experiment. Different experimental media were maintained in plastic 60-ml syringes connected to an eight-point manifold perfusate selector (Hamilton, Reno, NV) via one-way stopcocks and polyethylene tubing (Hyde et al., 2004). The rate (0.4 ml/min) of perfusion through the chamber was maintained by keeping the height of the syringes and volume of all solutions in the syringes constant throughout the experiment. In all experiment the chicken GnRH-II (cGnRH-II; Bachem, Torrance, CA) form was used since this form GnRH has been found to be most effective in inducing PRL release from tilapia RPDs (Weber et al., 1997). In experiments where U-73122 (Calbiochem, La Jolla, CA) was tested, all treatments contained the appropriate solvent (0.1% DMSO). Nifedipine was obtained from Sigma (St. Louis, MO) and 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) from Calbiochem.

Ratio images (excitation at 340 and 380 nm) were captured at 10 s intervals using an ORCA-1 cooled CCD camera (Hamamatsu, Welwyn Garden City, UK). Simultaneous measurements of intensities of free fura-2 (380 nm) and fura-2 bound to Ca^{2+} (340 nm) were analyzed with MetaFluor software (version 6.2r2; Universel Imaging). All data are expressed, as relative intensity of fura-2 fluorescence excited at 340 nm to that excited at 380 nm both with the background subtracted. Shifts in this ratio (340/380) result directly from changes in $[Ca_i^{2+}]$ (Grynkiewicz et al., 1985). Responsiveness to high-$[K^+]$ at the end of experimental runs was used as an indicator of cell viability.

2.4. IP_3 assay

Tilapia RPDs were pre-incubated in hyperosmotic medium for 2 h. After stimulation for 5 or 15 min, RPDs were harvested, placed in 100 μl ice-cold 4% perchloric acid, homogenized with a polypropylene homogenizer (Kontes, Vineland, NJ) frozen in liquid nitrogen and then stored at −80 °C. To retard degradation of IPs, 20 mM LiCl was included in the media 10 min before, and with, addition of cGnRH-II. On the day of the assay samples were thawed on ice and precipitated proteins were pelleted by centrifugation at 4 °C for 15 min at 2000*g*. The supernatant was transferred to a clean tube and adjusted to pH 7.5, with 1.5 M KOH/60 mM Hepes buffer containing universal pH indicator dye (0.5 ml/10 ml of buffer; Fisher, Pittsburgh, PA). Fifty microliters of neutralized sample was assayed in duplicate for IP_3 content with the $[^3H]IP_3$ receptor competitive binding assay system (Amersham Life Science, Newark, NJ). The precipitated proteins from the first centrifugation were dissolved in 0.1 M NaOH and protein content was measured according to Lowry et al. (1951). The IP_3 content was calculated as pmol/mg protein.

2.5. Statistics

Statistical differences were analyzed by using JMP 5.1.1 (SAS, Cary, NC). A two-factorial ANOVA was used to analyze overall effects of GnRH (with or without GnRH; factor 1) and co-treatment or sampling time (factor 2). When necessary, transformation of data were done to meet the ANOVA assumption of homogeneity of variances (evaluated by residual plots). When interaction between the two factors was significant post hoc test was performed. A priori it was decided to compare factor 2 vehicle control with co-treatment controls, and each co-treatment control with its GnRH counterpart. The differences were analyzed by Bonferroni adjusted least significant difference test for predetermined comparisons. In all cases, a significance level of $P = 0.05$ was used.

3. Results

3.1. Effect of GnRH on Ca_i^{2+} in PRL cells; interaction with a PLC inhibitor

In the Ca_i^{2+} imaging studies we used dispersed RPD cells (95–99% PRL cells) and the calcium-sensitive dye fura-2. We found that cGnRH-II-induced a rapid dose-dependent increase in Ca_i^{2+} in tilapia lactotrophs at concentrations ranging from 0.1 to 10 nM (see Fig. 1). The lowest cGnRH-II concentration that elicited the maximal response was 10 nM, and this concentration was used in following experiments. Because intracellular calcium mobilization may be caused by the activation of PLC, we undertook studies to evaluate if the cGnRH-II response on Ca_i^{2+} might be dependent on PLC-dependent phosphoinositide hydrolysis. As shown in Fig. 2, pretreatment of PRL cells with the membrane-permeable PLC-inhibitor, U-73122, resulted in the complete inhibition of the cGnRH-II-induced Ca^{2+} signal. In most cells spontaneous oscillations in Ca_i^{2+} was apparent, and PLC inhibition impeded these transients and made the cells quiescent (data not shown).

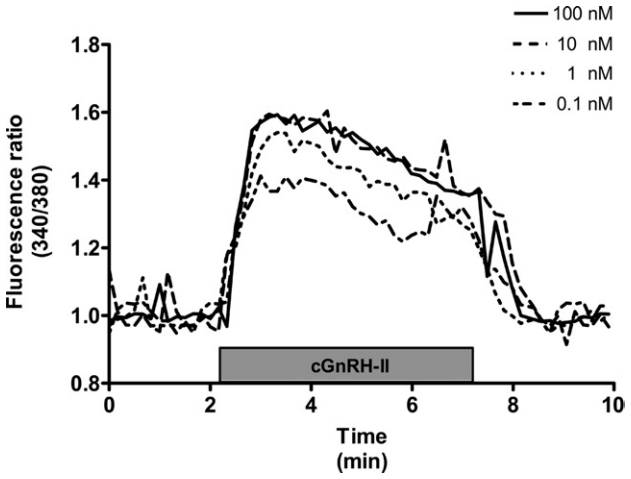

Fig. 1. Effect of different concentrations of cGnRH-II on Ca_i^{2+} in PRL cells expressed as the relative intensity of fura-2 fluorescence (340/380 nm). The magnitude of Ca_i^{2+} increase is correlated positively with cGnRH-II concentration. The block indicates the 5 min exposure time to cGnRH-II. Results are mean fluorescence ratio of cells ($n = 45$) from a representative experiment. The experiment was replicated in three separate incubations (different days) with similar results.

3.2. Effect of PLC inhibitor on GnRH-induced PRL-release

From the previous data, it would be expected that inhibition of PLC would uncouple GnRH-induced PRL release, if increased Ca_i^{2+} is causally coupled to exocytosis as widely believed. In an experiment employing static tissue incubations we tested if inhibition of PLC hinders cGnRH-II-induced PRL release. A 3-fold increase in $tPRL_{188}$ release from RPDs was seen in response to cGnRH-II, during 4 h incubation. The sustained cGnRH-II-induced $tPRL_{188}$ secretion was impeded by inhibition of PLC with U-73122 (Fig. 3). However, incubating the RPDs with U-73343, an inactive analog of U-73122 had no effect on the cGnRH-II evoked response, demonstrating the specificity of the observed effect. There was no significant difference between vehicle control and U-73122 or U-73343 controls.

3.3. Effect of GnRH on IP_3 levels

From the previous data, it would be expected that cGnRH-II possibly stimulates the activity of PLC in events that lead to increased Ca_i^{2+} and PRL release. Therefore, we next examined whether cGnRH-II could stimulate PLC activation and the subsequent production of IP_3. The IP_3 levels were measured in static incubations of RPDs in the presence of LiCl, an inhibitor of inositol phosphatases. As shown in Table 1, we found that cGnRH-II induces a rapid (<5 min) and sustained increase in the level of IP_3 in tilapia RPDs ($P < 0.01$).

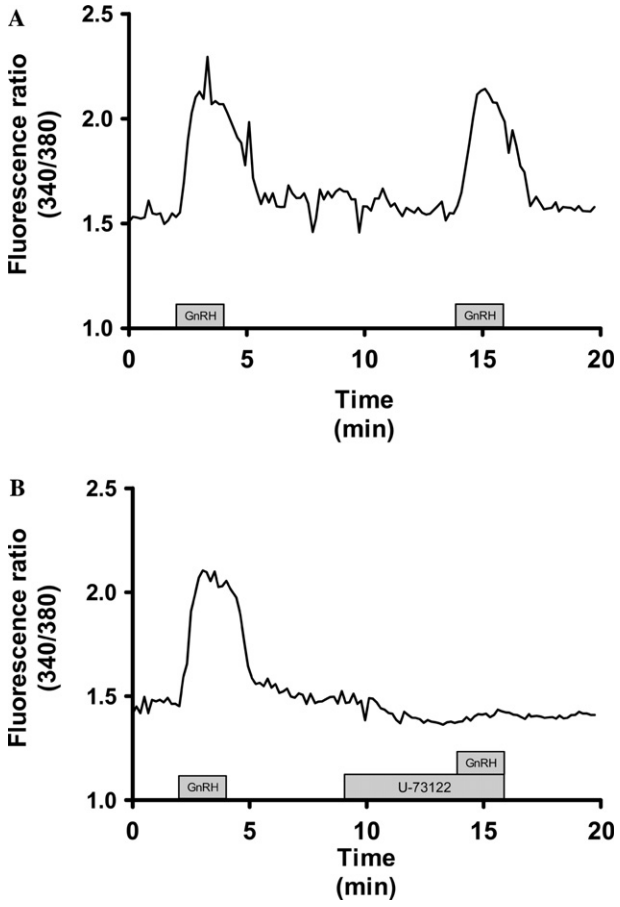

Fig. 2. Effects of PLC inhibitor (U-73122; 4 μM) on Ca_i^{2+} mobilization induced by cGnRH-II (10 nM). On the control coverslip, PRL cells were exposed two times to cGnRH-II (2 min) with a 10 min gap between exposures (A). On a second coverslip the second cGnRH-II exposure was preceded by 5 min treatment with U-73122 (B). The Ca_i^{2+} increase in response to cGnRH-II was blocked by the PLC inhibitor. Results are mean fluorescence ratio of cells ($n = 42$ and 38 for control and U-73122 coverslip, respectively) from representative experiments performed the same day. The experiment was repeated with four different cultures, and in all cases U-73122 blocked the response to cGnRH-II.

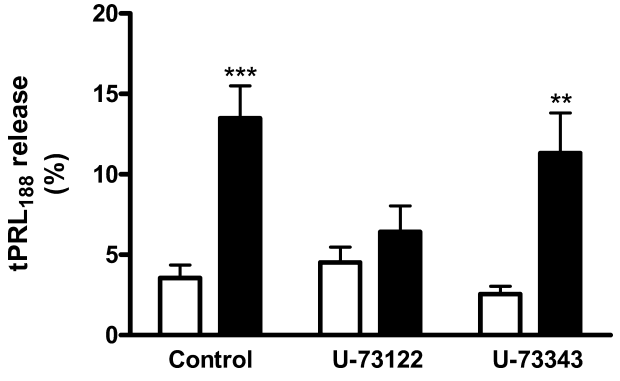

Fig. 3. Effects of control media (open bars) and cGnRH-II (solid bars; 10 nM) combined with control media, U-73122 (4 μM) or U-73343 (4 μM) on release of $tPRL_{188}$ from the RPD of male tilapia. After 2 h pre-incubation, RPDs were incubated with the agents for 4 h. Values are means ± SEM ($n = 8$). Asterisks indicate significant difference from respective control (**$P < 0.001$; ***$P < 0.001$).

Table 1
Inositol triphosphate content (pmol/mg protein) in RPDs were determined by a radioreceptor assay in the presence of 20 mM LiCl

	Control	cGnRH-II [a]
5 min	73.8 ± 16.7	136.4 ± 18.8
15 min	78.0 ± 9.3	122.6 ± 12.2

Tissues were treated with cGnRH-II (10 nM) for the indicated time periods. Values are means ± SEM ($N = 6$; three tissues each).

[a] Significant overall effect of cGnRH-II analyzed by two-way ANOVA ($P \leqslant 0.01$).

3.4. Effect of TMB-8 on GnRH-induced Ca_i^{2+} signals

In a series of experiments we tested the effect of a broad-spectrum blocker of Ca^{2+} release from intracellular stores (TMB-8) on GnRH-induced increase in Ca_i^{2+}. As shown in Fig. 4, TMB-8 totally blocks the response to cGnRH-II. After washout the cells become sensitive to the hormone again. TMB-8 itself rapidly induces a fluctuation in Ca_i^{2+} when first applied (or removed) followed by establishment of a new baseline. Cells with spontaneous oscillations in Ca_i^{2+}, lose these transients during the TMB-8 exposure, but regain them after a washout (Fig. 4B).

3.5. Effect inhibitors of Ca_i^{2+} signaling on sustained GnRH-induced PRL-release

In this experiment an inhibitor of release of Ca_i^{2+} from intracellular stores, TMB-8, and of Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC), nifedipine, were employed (Fig. 5). TMB-8 blocked the effect of cGnRH-II on $tPRL_{188}$. Inhibition of VGCC by nifedipine, also inhibited the sustained response to cGnRH-II. There was no significant difference between vehicle control and TMB-8 or nifedipine controls.

4. Discussion

This study is the first to examine the signaling pathways that mediate GnRH-evoked PRL secretory responses in vertebrate lactotrophs. It is known that exocytosis in general require Ca_i^{2+} in all model systems studied thus far (Kasai, 1999). In tilapia previous studies have shown that PRL release from the tilapia RPD increases with a rise in Ca_i^{2+} (Borski et al., 1991). In the present investigation we found that cGnRH-II increases Ca_i^{2+} in a dose-dependent fashion. This suggests that a hormone–receptor interaction induces the increase in Ca_i^{2+} leading to PRL release, in accordance with the finding that GnRH is a potent dose-dependent stimulator of PRL release in the tilapia (Weber et al., 1997).

In goldfish somatotrophs and gonadotrophs cells intracellular Ca^{2+} stores are involved in GnRH-induced Ca^{2+}/secretion coupling (Johnson and Chang, 2000; Johnson et al., 2000). In the present study we demonstrate that GnRH-II-induced Ca^{2+}/secretion coupling in PRL cells is dependent on PLC activation, since U-73122 blocks increases in Ca_i^{2+} levels and PRL release elicited by GnRH. Thus, in the lactotroph the secretory response may involve receptor-mediated activation of PLC, generation of the second messengers IP_3 and DAG leading to mobilization of intracellular pools of Ca^{2+} and activation of PKC.

We found, that inhibition of the release of Ca^{2+} from intracellular stores with TMB-8 also abolished both the cGnRH-II-induced response in Ca_i^{2+} (Fig. 4) as well as

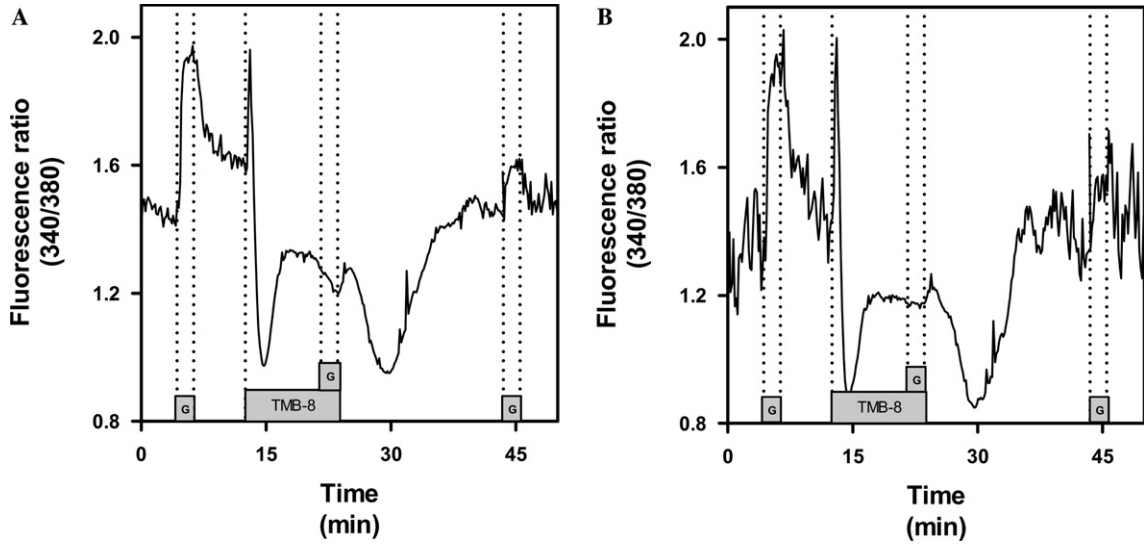

Fig. 4. Effects of TMB-8 (a blocker of intracellular Ca^{2+} release; 100 μM) on Ca_i^{2+} mobilization induced by cGnRH-II (10 nM). The initial response to cGnRH-II (marked with G) is abolished by TMB-8 (5 min pre-treatment). PRL cells regain sensitivity to cGnRH-II following washout of TMB-8. (A) The mean fluorescence ratio of cells ($n = 36$) from a representative experiment is shown. (B) A single cell is shown. The experiment was repeated with four different cultures and in all cases TMB-8 blocked the response to cGnRH-II.

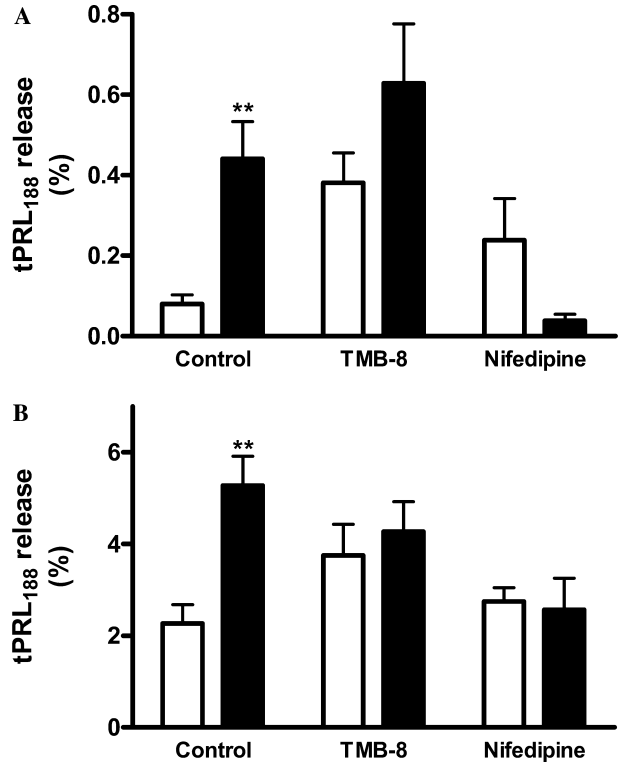

Fig. 5. Effects of control media (open bars) and cGnRH-II (solid bars; 10 nM) combined with control media, TMB-8 (100 μM), or nifedipine (Nif; 10 μM) on release of $tPRL_{188}$ from the RPD of male tilapia. After 2 h pre-incubation, RPDs were incubated with the treatment media for 0.5 (A) and 4 h (B). Values are means ± SEM ($n = 8$). Asterisks indicate significant difference of cGnRH-II treatment from respective control (**$P < 0.01$).

sustained PRL release (Fig. 5). In goldfish gonadotrophs GnRH-induced Ca^{2+} signaling is inhibited by TMB-8, and both IP_3-sensitive and -insensitive intracellular stores is thought to be involved (Johnson et al., 2000). Since both U-73122 and TMB-8 blocks cGnRH-II-induced Ca^{2+} signaling and PRL release in tilapia lactotrophs and cGnRH-II increase the cellular content of IP_3, it suggests the involvement of IP_3 sensitive intracellular Ca^{2+} pools. Interestingly both U-73122 and TMB-8 also dampened spontaneous Ca_i^{2+} transients indicating that IP_3 sensitive stores of intracellular Ca^{2+} is critical for the activity of cells that exhibit oscillating Ca_i^{2+}. This agrees with the observation that IP_3 seems to be essential for the generation of intracellular Ca^{2+} oscillations in gonadotrophs (Hille et al., 1995), and is in agreement with the findings that TMB-8 sensitive Ca^{2+} stores are involved in the spontaneous Ca^{2+} signaling in goldfish somatotrophs (Johnson and Chang, 2000).

Taken together, these results indicate that the signaling pathways that mediate GnRH-induced PRL release in tilapia lactotrophs, require Ca^{2+} release from IP_3/TMB-8 sensitive intracellular stores. Similarly in goldfish somatotrophs, GnRH-induced Ca^{2+} signals and GH release seems dependent on intracellular Ca^{2+} pools (Johnson and Chang, 2000). That the response to GnRH in lactotrophs involve PKC activation by DAG and increased $[Ca^{2+}]_i$, cannot be excluded. Inhibition of VGCC by nifedipine abolishes sustained GnRH stimulated PRL release (0.5, 4 h). This might seem somewhat paradoxical, as it indicates that the agonist-induced PRL secretion is completely dependent on intracellular stores, as well as, influx of extracellular Ca^{2+}. However, the question remains whether VGCC activation initiates the Ca^{2+} signal leading to secretion or is secondary to release of Ca^{2+} from intracellular stores, as suggested for somatotrophs (Johnson and Chang, 2000). In that case, the role of VGCC-mediated Ca^{2+} influx would be to replenish intracellular Ca^{2+} stores since Ca^{2+} influx is ultimately necessary for maintaining intracellular/IP_3-sensitive stores. Future studies with measurement of acute PRL release (<30 min) could prove helpful in resolving this question. Nevertheless, the fact that U-73122 and TMB-8 fully inhibit the GnRH-induced Ca^{2+} signal, indicates that VGCC activation is downstream to the release of Ca^{2+} from intracellular stores.

The present study is the first to report that GnRH acts to induce PRL release from a fish lactotroph by increasing Ca_i^{2+}. Furthermore, the rise in Ca_i^{2+} may be derived from PLC/IP_3-induced mobilization of Ca^{2+} from intracellular stores along with influx through L-type channels. It has been shown that GnRH neurons innervate the tilapia pituitary (Yamamoto et al., 1998), and an isoform of the GnRH receptor has been found to co-localize with PRL in the RPD of this species (GnRH-R type 1B; Parhar et al., 2002). These findings, along with the present study all point to GnRHs physiological role as a regulator of PRL secretion in tilapia.

Acknowledgments

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